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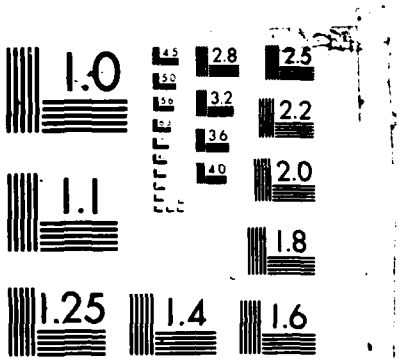

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Molecular Modeling in Drug Design for the Development of Organophosphorus Antidotes/Prophylactics

Annual Report

Wah Chiu, Ph.D.

April 30, 1985

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Our long-term goal is to design drug against the acetylcholinesterase inhibitors by molecular modeling techniques and theoretically predicted action of drug. Prior to this effort, we need to have more knowledge of the three-dimensional strucutre of the acetylcholinesterase. In the current year, we have tested various biochemical procedures for purifying acetylcholinesterase from electrical eel in the form suitable for structural analysis by electron diffraction method. Preliminary result suggests the feasibility of crystallizing the acetylcholinesterase. New computer algorithm has been implemented for processing electron micrograph data for structural investigation.

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Summary

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Foreword

Citations of commerical organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

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Annual Report for May 1, 1984 - April 30, 1985

Contract No. DAMD 17-84-C-4110

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University of Arizona, Tucson, AZ 85721

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Term of Contract: May 1, 1984 - April 30, 1987

Principal Investigator: Dr. Wah Chiu
Telephone No. (602)-621-7524 or 621-1773

(I) Purification and preliminary structural studies of electric eel acetylcholinesterase

Introduction

Acetylcholinesterase is an enzyme which plays an integral role in the function of cholinergic synapses. This enzyme catalyzes the hydrolysis of the neurotransmitter acetylcholine to choline and acetic acid.

Several molecular forms of acetylcholinesterase have been identified. Asymmetric forms have been identified in the electric organs of Torpedo and Electrophorus and in mammalian muscle and brain (Dudai et al., 1973; Rieger et al., 1973; Lwebuga-Mukasa et al., 1976; Rieger et al., 1980). These molecules consist of clusters of globular catalytic subunits which are anchored to the basement membrane by a collagen-like tail. At least three different sizes differing in the number of catalytic subunits have been identified by density gradient centrifugation.

A second form of the enzyme has been identified in Torpedo electric organ (Bon and Massoulie, 1980; Viratelle and Bernhard, 1980; Lee et al., 1982) and mammalian erythrocyte membranes (Grossman and Lieflander, 1975; Ott et al., 1975) . This form of the enzyme consists of a disulfide linked dimer of catalytic subunits which is anchored to the plasma membrane. Early evidence indicated that a small hydrophobic peptide anchored the esterase to the membrane, but recent evidence suggests that the enzyme is linked directly to phosphatidylinositol (Stieger et al., 1984; Stieger and Brodbeck, 1985; Futerman et al., 1985).

The structural information available on acetylcholinesterase is limited to biochemical characterizations and several electron micrographs (for review see Rosenberry, 1975; Massoulie and Bon, 1982). Density gradient centrifugation and electron microscopy established the asymmetric nature of the 9S, 14S, and 18S forms and the apparent tetrameric nature of the 11S form produced by proteolysis of the asymmetric forms (Dudai et al., 1973; Rieger et al., 1976). Circular dichroism studies and an amino acid analysis revealed the collagen-like nature of the tail fragment of the asymmetric forms (Mays and Rosenberry, 1981). Amino acid analysis of the globular 11S form has also been reported (Leuzinger and Baker, 1967; Bon et al., 1976). Peptide mapping has demonstrated that the catalytic subunits of the dimeric membrane form and the globular 11S form are different (Lee et al., 1982). Crystallization of the 11S form and preliminary X-ray diffraction results have been reported, but no structural information has been obtained (Leuzinger et al., 1968; Chothia and Leuzinger, 1976).

Experimental Methods

Numerous acetylcholinesterase purification schemes have been published. Nearly all involve affinity purification using quaternary ammonium esterase inhibitors as the affinity ligand (Berman and Young, 1971; Massoulie and Bon, 1976; Webb and Clark, 1978; Ralston et al., 1983). The methods developed by these investigators provided the groundwork from which we have developed our purification procedure. We have so far focused our effort in the purification and structural determination of the 11S form of the enzyme.

The purified enzyme is evaluated by SDS and nondenaturing polyacrylamide gels. Electron microscopy of the purified enzyme was done initially in a Hitachi electron microscope in the College of Agriculture and subsequently in our newly acquired Philips 420 electron microscope.

We have set up several crystallization conditions of different pH, ionic strength and protein concentration with our purified AChE (McPherson, 1976). Light microscopy was used to monitor the growth of the crystals.

We have been developing a set of computer programs for 3 dimensional reconstruction of noncrystalline objects and of objects with a helical symmetry. We expect to use these computer algorithms to determine the molecular structures of the catalytic subunits of acetylcholinesterase and of the tail part of this enzyme which appears to have a helical arrangement.

Results

Crystallization of proteins for structural determination requires milligram quantities of pure protein (McPherson, 1976). These quantities of acetylcholinesterase are available commercially and, therefore, several of these preps were evaluated. Sigma, Worthington, and Boehringer Mannheim esterase preparations of 11S AchE were analyzed on both SDS and nondenaturing polyacrylamide gels. Multiple bands were observed in all of these preps, even after further purification of the esterase by affinity chromatography in our laboratory. Figures 1a and 2b show the banding pattern observed from a Sigma prep. The SDS gel pattern suggests some proteolytic degradation during the preparation by Sigma.

In order to better control the treatment of the esterase, we developed a purification procedure using electric organ from Electrophorus electricus as the starting material. This procedure involves affinity purification and trypsinization of the asymmetric forms to produce a more soluble globular 11S form. The best preparation we have obtained shows two major bands in a polyacrylamide gel stained for esterase activity (Figure 2a). The SDS gel pattern of the same prep shows a single major band around 70,000 daltons (Figure 1b). The performance of the affinity columns has been found to be somewhat erratic. We have found that contaminating proteins sometimes co-purify with the esterase. Some of the heterogeneity observed in our preps by gel electrophoresis (as in Figure 1c) may be the result of nonuniform tryptic cleavage. Standardization of the tryptic digestion procedure and optimization of our affinity column performance will alleviate these problems. Progress is being made in this regard. Our purification protocol should be capable of providing the quantities of pure acetylcholinesterase of 11S form needed for the structural studies.

We examined some of our purified enzyme of 11S in the electron microscope. Figure 3 is a typical micrograph of this preparation and shows various sizes. These may represent various types of aggregates of the monomer. More work is needed to sort out whether the aggregation is inherent to this form of the enzyme or whether it represents an artifact resulting from specimen preparation techniques.

In our preliminary trials, we have obtained several crystal forms of 11S AchE as shown in the light microscope. Figure 4 shows the largest crystal to be 300 microns on one edge of the crystal. Unfortunately, this crystal form is too big for electron diffraction and too small for X-ray diffraction analysis. We are certain that this crystal is formed by the enzyme because we have run an activity gel of the protein prior to its use for crystallization. We plan to refine the crystallization procedure to make the crystal suitable for electron diffraction analysis.

We have completed the implementation of the computer program for reconstructing the 3 dimensional structure of an object with a helical symmetry. We tested this program initially with simulated data and subsequently with an electron micrograph of a T4 phage tail which is known to have helical symmetry (DeRosier and Klug, 1968). The reconstruction of T4 phage tail agrees well with the published results. This confirms the correctness of our computer algorithm. We also used this set of programs to test a presumably helical object of unknown structure. The reconstruction result looks quite promising. With this algorithm providing the groundwork, we will develop in the next year the computer software necessary for the 3-dimensional reconstruction of an object with no crystallographic symmetry.

Conclusion

In summary, we have accomplished our proposed task in year 01 to demonstrate our capability of purifying the eel acetylcholinesterase, to record a preliminary electron image of this enzyme and to show the feasibility of forming a crystal from this enzyme.

(II) Installation of Major Equipment

We have installed two major instruments which are essential for the proposed research. The first instrument is the Perkin Elmer 1010M microdensitometer which was installed in July 1984. The instrument was tested by our professional staff to be performing as specified by the manufacturer. We have used it to digitize our electron microscopic data prior to its processing by computer. The second instrument is the Philips 420 electron microscope which was installed in March 1985. This equipment is performing as specified at room temperature. We are waiting for the delivery of the specimen rotation holder and the cold specimen holder from the manufacturer around midsummer 1985.

(III) Research Plan for Year 02 (May 85 - April 86)

We plan to refine our biochemical preparation procedures to make it a reliable and reproducible one, to scale up for the production of a large quantity of this enzyme for structural study, and to refine the crystallization condition of this enzyme. We will try to optimize the tryptic treatment of the whole enzyme. We plan to improve the affinity column used for the purification step. We will try to use different columns as suggested by the COTR and some other investigators (Ralston et al., 1983; Webb and Clark, 1978). The variations of the affinity resins to be tested include different sizes of spacer arms and different types of ligands. For example, procainamide and acridinium will be used as ligands in the column. The possible spacer arms for trial are diaminodipropylamine, hexanediamine and aminocaproic acid-diaminopropane. We plan to continue the development of computer software for reconstructing 3 dimensional structure of individual protein molecules. We anticipate collection of preliminary 3 dimensional data of this enzyme either in noncrystalline or in crystalline form with our newly acquired electron microscope.

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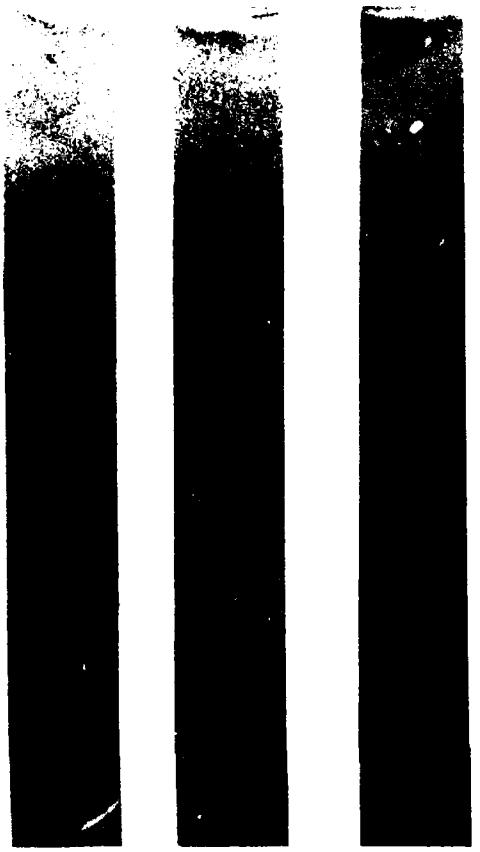
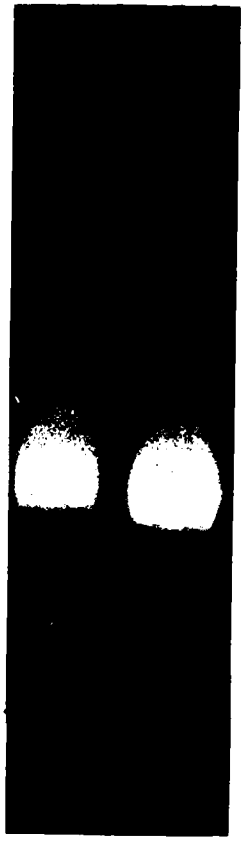


1a 1b 1c

Figure 1



2a 2b

Figure 2

Figure 1: SDS-Polyacrylamide Gel Electrophoresis of Electric Eel AChE. Polyacrylamide gel stained for protein with Coomassie Blue. Lane a: AChE (Sigma) after further purification; specific activity = 4000 U/mg. Lane b: AChE prepared from electric organ (this laboratory); specific activity = 4000 U/mg. Lane c: Second preparation of AChE from electric organ (this laboratory); specific activity = 2000 U/mg. 1 Unit = 1 mole acetylthiocholine/min.

Figure 2: Native Electrophoresis of Electric Eel AChE. Polyacrylamide gel stained for acetylcholinesterase activity. Lane a: AChE prepared from electric organ in this laboratory. Lane b: AChE purified in this laboratory starting from a commercial preparation (Sigma).

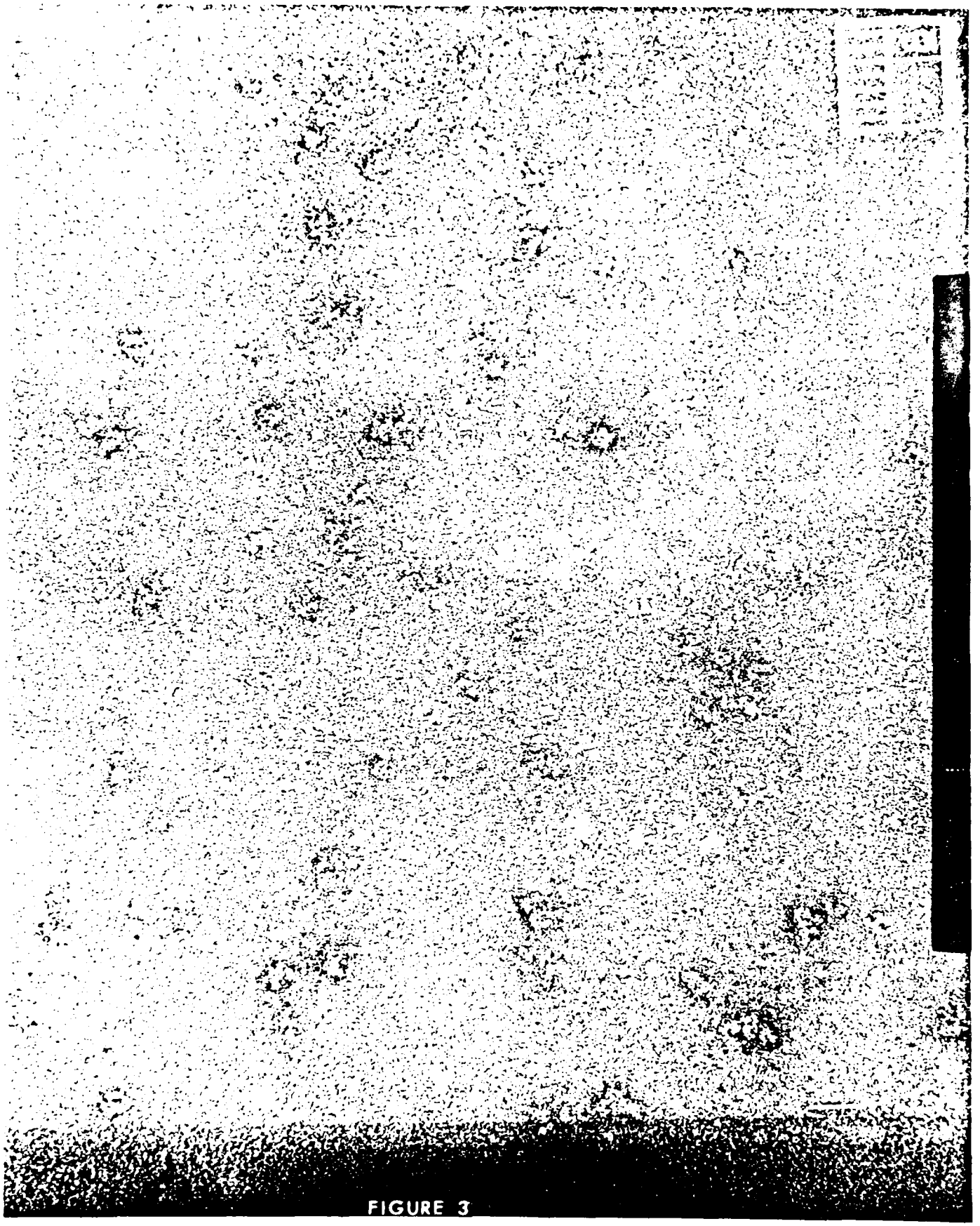


FIGURE 3

Figure 4: Light Micrograph of Acetylcholinesterase Crystals.

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